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MSH2 is required for cell proliferation, cell cycle control and cell invasiveness in colorectal cancer cells

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We have investigated the role of *MSH2*, a mismatch repair gene in cell proliferation, cell cycle control and cell invasiveness in the SW480 human colorectal cancer cell line. RNAi-mediated inhibition of *MSH2* expression was achieved using *MSH2* shRNA lentiviral expression vectors. Effective knockdown of endogenous *MSH2* expression was determined by real-time PCR analysis. The most efficient *MSH2* knockdown vector was selected for subsequent studies using SW480 cells. Endogenous *MSH2* mRNA levels decreased after lentiviral delivery of the MSH2-RNAi, indicating efficient silencing of *MSH2* expression in SW480 cells. Cell proliferation, cell cycle progression and cell invasiveness were quantified by MTT assays, flow cytometry and transwell assays, respectively. RNAi-mediated inhibition of *MSH2* expression in SW480 cells resulted in decreased cell proliferation, cell cycle arrest at the G0/G1 phase and decreased cell invasiveness. Taken together, these results provide evidence that MSH2 stimulates cell proliferation, promotes cell cycle progression and positively regulates cell invasiveness.

colorectal cancer, hereditary nonpolyposis, RNAi, MSH2

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Colorectal carcinoma (CRC) is one of the most common malignant tumors of the gut, and it has been associated with increasing morbidity in China in recent years (http://seer.cancer.gov). Hereditary nonpolyposis colorectal carcinoma (HNPCC, Lynch syndrome) is the most common hereditary form of CRC, and it is believed to account for between 5%–10% of the total CRC burden [1]. HNPCC is characterized genetically by pathogenic germline mutations in DNA mismatch repair (MMR) genes, most often in the *MLH1* and *MSH2* genes [2]. Recent studies have shown that MSH2 plays an important role, not only in mismatch repair, but also in cell proliferation, apoptosis and cell cycle control [3].

MSH2 is a component of the highly conserved post-replicative DNA MMR system. DNA MMR proteins contribute to DNA replication fidelity by removing insertion or deletion loops that result from template primer slippage at repetitive DNA sequences, and by correcting single base mismatches that escape polymerase proofreading, thereby preventing the accumulation of spontaneous mutations and ensuring the integrity and stability of the genome [4,5].

MSH2 has been shown to play an important role in the regulation of cell proliferation. Srivastava et al. [6] reported that MSH2 protein levels were significantly higher in 28 glioblastoma multiforme samples relative to 27 unpaired astrocytoma samples; in contrast, no such differences in MLH1 levels were observed.

Experimental evidence has shown a role for MMR genes in the regulation of apoptosis. Zhang et al. [7] found that over-expression of *MSH2* or *MLH1*, but not *MSH3*, *MSH6* or *PMS2*, induced apoptosis in either repair-proficient or repair-deficient cells, suggesting that the mismatch repair proteins MSH2 and MLH1 may be components of a pathway that influences apoptosis.

DNA damage can lead to genomic instability. Genomic instability is often inhibited following cell cycle arrest. G2 and S phase checkpoints and many associated proteins, including ATM, ATR, p53, p73, Chk1 and Chk2 [8–10] have

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shown important roles in the regulation of genomic stability.

In the current study, we carried out *MSH2* RNAi experiments to investigate the role of MSH2 in colon cancer cell proliferation, cell cycle regulation and cell invasiveness.

1 Materials and methods

1.1 Cell culture

The SW480 cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. SW480 cells were infected with different lentiviral strains at an MOI of approximately 10–50 for 3–5 d, lysed and subjected to RT-PCR. All cell culture reagents were from Gibco.

1.2 MSH2 RNAi lentivirus generation

The full-length human *MSH2* mRNA sequence was submitted to the siRNA design center of Shanghai GeneChem Co. for the generation of four oligo sequences against *MSH2*. The *MSH2* RNAi target sequences included:

#1: 5′-AACCTTCATTTGATCCTAA-3′, starting at the +1479 site in the mature *MSH2* mRNA;

#2: 5′-CTTGAGGAGTTTCAGTATA-3′, starting at the +2618 site in the mature *MSH2* mRNA;

#3: 5′-AGGTGTCTGTGATCAAAGT-3′, starting at the +2524 site in the mature *MSH2* mRNA;

#4: 5′-GAAGATGCAGTCAACATTA-3′, starting at the +1537 site in the mature *MSH2* mRNA; and

non-target control: 5′-TTCTCCGAACGTGTCACGT-3′.

For the shRNA-expressing lentiviral vector, RNAi stem-loop DNA oligos were designed and synthesized by Shanghai GeneChem Co. (Table 1). The oligos were annealed and cloned into the lentivirus-based RNAi vector, pGCL-GFP (Addgene, Shanghai GeneChem Company, Shanghai, China), and a non-targeting stem-loop DNA was cloned into pGCL-GFP to generate the negative control lentiviral vectors. The lentiviral infecting particles were prepared as described previously [11]. Cells were infected with the MSH2-RNAi-lentivirus or with negative control virus when they were 30%–40% confluent, and were examined 2–8 d later. Briefly, 293T cells were co-transfected with the pGCL-GFP RNAi vector and packaging vectors, and the resulting supernatant was collected after 48 h. The virus was recovered after ultracentrifugation for 3.5 h at 25000 r/min in a Beckman SW28 rotor and resuspended in phosphate-buffered saline (15–200 μL). Titers were determined by infecting 293T cells with serial dilutions of concentrated lentivirus. We determined EGFP expression in infected cells by flow cytometry (FACSCalibur, BD, USA) at 96 h post-infection; in a typical preparation, the titer was approximately 1–2×10^8 infectious units (IFU) per mL.

Table 1 DNA sequences of the *MSH2* shRNAs

Marker	Gene	TargetSeq
1#-RNAi-LV	*MSH2*	AACCTTCATTTGATCCTAA
2#-RNAi-LV	*MSH2*	CTTGAGGAGTTTCAGTATA
3#-RNAi-LV	*MSH2*	AGGTGTCTGTGATCAAAGT
4#-RNAi-LV	*MSH2*	GAAGATGCAGTCAACATTA
NC-RNAi-LV		TTCTCCGAACGTGTCACGT

1.3 RT-PCR reactions

A TRIZOL Reagent kit (Invitrogen) was used to isolate mRNA from infected cells. First-strand synthesis and PCR reactions were performed using M-MLV reverse transcriptase and *Taq* polymerase (Promega), respectively, according to the manufacturer's instructions. The actin primer set included the hActin-F primer (5′-GTGGACATCCGCAAAGAC-3′) and the hActin-R primer (5′-AAAGGGTGTAACGCAACTA-3′). The human MSH2 primer set included the MSH2-F primer (5′-GCCATGTGAGTCAGCAGAAG-3′) and the MSH2-R primer (5′-CCCAAATCCATCGTAGGTAGAAG-3′). Quantification of the RT-PCR results was conducted using BioRadQ5 software, and statistical analyses were performed using a one-way ANOVA analysis. Differences with a P value of < 0.05 were considered statistically significant. Experiments with multiple experimental conditions were repeated at least three times.

1.4 Cell survival assay

SW480 cells were divided into three groups. Control group: normal SW480 cells; NC-RNAi-LV group: SW480 cells infected by the lentivirus without the specific interference RNA; MSH2-RNAi-LV group: SW480 cells infected by the lentivirus with the specific interference RNA. Cell survival and proliferation were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were plated in a 24-well plate at a density of 50000 cells/well and incubated overnight in the presence of 10% FBS. Cells were infected with different MSH2-RNAi lentiviruses for different periods of time. To examine cell survival/proliferation, a one-tenth volume of MTT (5 mg/mL in PBS, Sigma) was added to each well, and the plate was maintained for an additional 3 h in the incubator. After removing the medium, the insoluble metabolized MTT substrate was dissolved in acid-isopro- panol (100 μL/well, 0.07 mol/L HCl-isopropanol). The plates were rocked gently, and the absorbance of the supernatant was measured spectrophotometrically (Thermo Labsystems, MK3, USA) at 490 nm. The values obtained were proportional to the number of viable cells, and the results are expressed as the mean ± SEM values ($n > 3$). Cell survival was also estimated using flow cytometry. Propidium iodide (PI, Sigma) was added to a final concentration of 50 μg/mL 30 min before analyzing the cell cycle profile by flow cytometry. The percentages of cells in different cell cycle phases are expressed as the mean ± SEM ($n > 3$). Cell inva-

sion was measured by an inclined test using polycarbonate filters incorporated into modified Transwell chambers. Cells stained with crystal violet (purple colored cells) indicated the number of cells that had migrated across the membrane. The migration rate of SW480 cells was calculated by the ratio of migrated cells/total cells (the migrated cells were dissolved in 10% acetic acid and measured spectrophotometrically at 570 nm).

1.5 Statistical analyses

One-way ANOVA analysis was used to evaluate the differences among the three groups: control, NC-RNAi-LV and MSH2-RNAi-LV in respects of cell proliferation, cell cycle control and cell invasiveness. SPSS Version 13.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses. $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 Efficient knockdown of MSH2 expression in SW480 cells

Four pairs of RNAi targeting sequences were synthesized and inserted into the lentiviral expression vector pGCL-GFP, as shown in Figure 1(a) and Table 1. Initial experiments to screen the RNAi target sequences were performed using 293T cells, which were infected with control (NC-RNAi-LV) or one of the four MSH2-RNAi lentiviral particles. Infected 293T cells were allowed to grow for 4 d before total RNA was extracted. Percentage infection was monitored by light microscopic observation of the ratio of EGFP-positive cells relative to the total number of cells; in these experiments, more than 80% of cells were infected by lentivirus (Figure 1(b), left panel). Infection of 293T cells with lentiviruses expressing MSH2-RNAi #2, #3 or #4 led to effective knockdown of endogenous MSH2 expression, as determined by RT-PCR analysis. MSH2-RNAi #3 showed the most efficient MSH2 knockdown (the knockdown efficiency was 55%), and was selected for subsequent studies using SW480 cells (Figure 1(b), right panel). Taken together, these data suggested that the lentiviral vector-based MSH2-RNAi effectively inhibited endogenous *MSH2* expression.

As the MSH2-RNAi #3 vector most effectively inhibited MSH2 expression in 293T cells, we carried out experiments to determine whether it also silenced endogenous *MSH2* expression in SW480 cells. We infected SW480 cells with

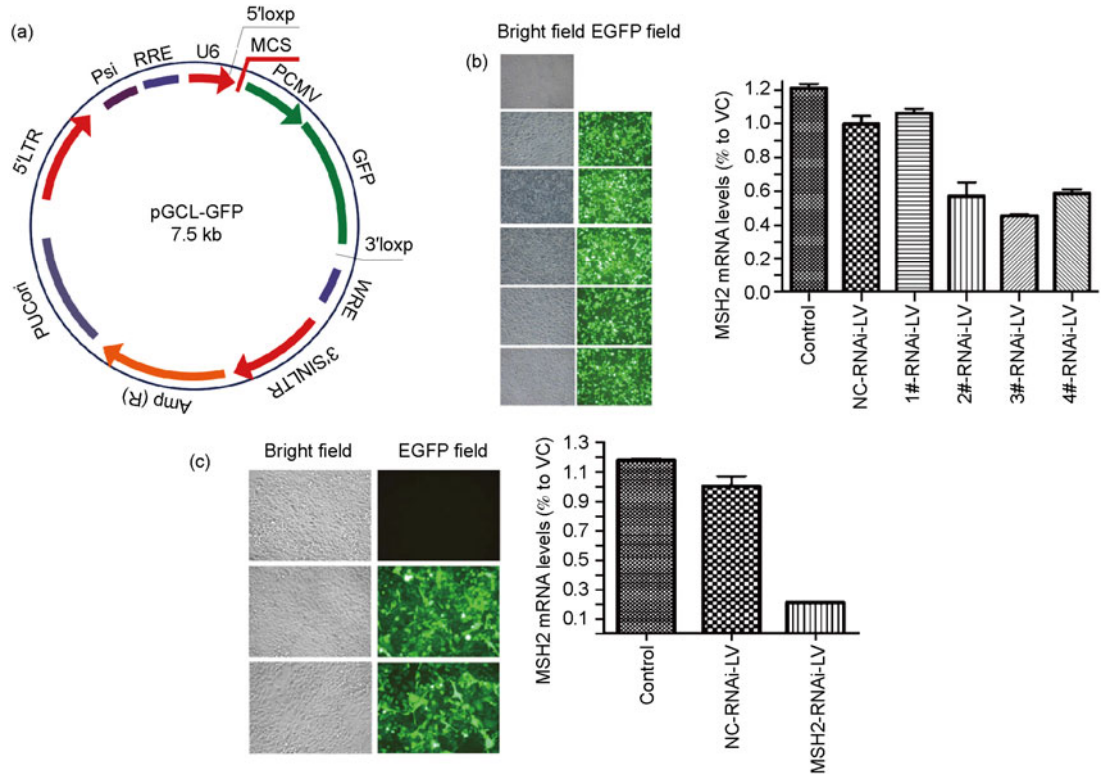

Figure 1 Summary of *MSH2* RNAi experiments. (a) Generation of a lentiviral vector for expression of *MSH2* shRNAs. The pGCL vector was engineered by introducing the mouse U6 promoter upstream of a PCMV-EGFP expression cassette to generate a vector that simultaneously produces shRNA and the EGFP reporter gene. CMV, cytomegalovirus promoter; WRE, woodchuck hepatitis virus response element. (b) Specific degradation of *MSH2* mRNA induced by different MSH2-RNAi lentiviral vectors. EGFP expression levels in infected 293T cells (left). Bar graphs represent average *MSH2* expression levels (%) relative to a vehicle control (VC) (right). Error bars represent the SEM. All samples were normalized to actin expression. (c) Functional silencing of *MSH2* expression in SW480 cells infected with an MSH2-RNAi lentiviral vector and control vectors. EGFP expression levels in SW480 cells infected with MSH2-RNAi lentiviral and control vectors (left). Bar graphs represent average *MSH2* expression levels (%) relative to a VC (right). Error bars represent the SEM. All samples were normalized to actin expression.

either NC-RNAi-LV or MSH2-RNAi #3 lentivirus (MSH2-RNAi-LV). Total RNA was extracted from SW480 cells at 4 d post-infection, and was subjected to RT-PCR analysis to evaluate endogenous *MSH2* mRNA levels. Endogenous *MSH2* mRNA expression decreased after lentiviral delivery of the MSH2-RNAi (Figure 1(c)) (the knockdown efficiency was 80%), showing efficient silencing of *MSH2* expression in SW480 cells.

2.2 RNAi-mediated silencing of MSH2 expression in colon cancer cells led to decreased cell proliferation, a G0/G1 cell cycle arrest and reduced cell invasiveness

To determine whether inhibition of *MSH2* expression in colon cancer cells may influence tumor cell proliferation, we performed MTT assays to study cell proliferation. SW480 cell proliferation rates began to decrease 72 h after RNAi-mediated *MSH2* silencing. At both 72 and 120 h after RNAi-mediated *MSH2* silencing, we noted significantly lower rates of cell proliferation relative to control cells (Figure 2(a), Table 2). This result showed that the inhibition of *MSH2* expression in colon cancer cells led to decreased cell proliferation, and provided evidence that *MSH2* may play an important role in the regulation of colon cancer cell proliferation *in vivo*.

We also used flow cytometry to study cell cycle progression following RNAi-mediated inhibition of *MSH2* expression. Five days after RNAi-mediated *MSH2* silencing, SW480 cells were stained with PI and analyzed by flow cytometry. The results of these experiments showed that decreased *MSH2* expression led to cell cycle arrest in the G0/G1 phase (Figure 2(b), Table 3). These data suggested that MSH2 may play an important role in the G1/S checkpoint.

Table 2 Cell proliferation in colon cancer cells after RNAi-mediated silencing of *MSH2* expression (MTT assays)[a)]

Day	Control (*A*)	NC-RNAi-LV (*A*)	MSH2-RNAi-LV (*A*)	*P*
1	0.056±0.003	0.055±0.003	0.062±0.009	0.329
2	0.173±0.005	0.149±0.002	0.178±0.009	0.343
3	0.444±0.026	0.434±0.028	0.353±0.018	0.007
4	0.755±0.125	0.695±0.032	0.588±0.037	0.095
5	0.790±0.072	0.784±0.035	0.654±0.039	0.030

a) $P < 0.05$.

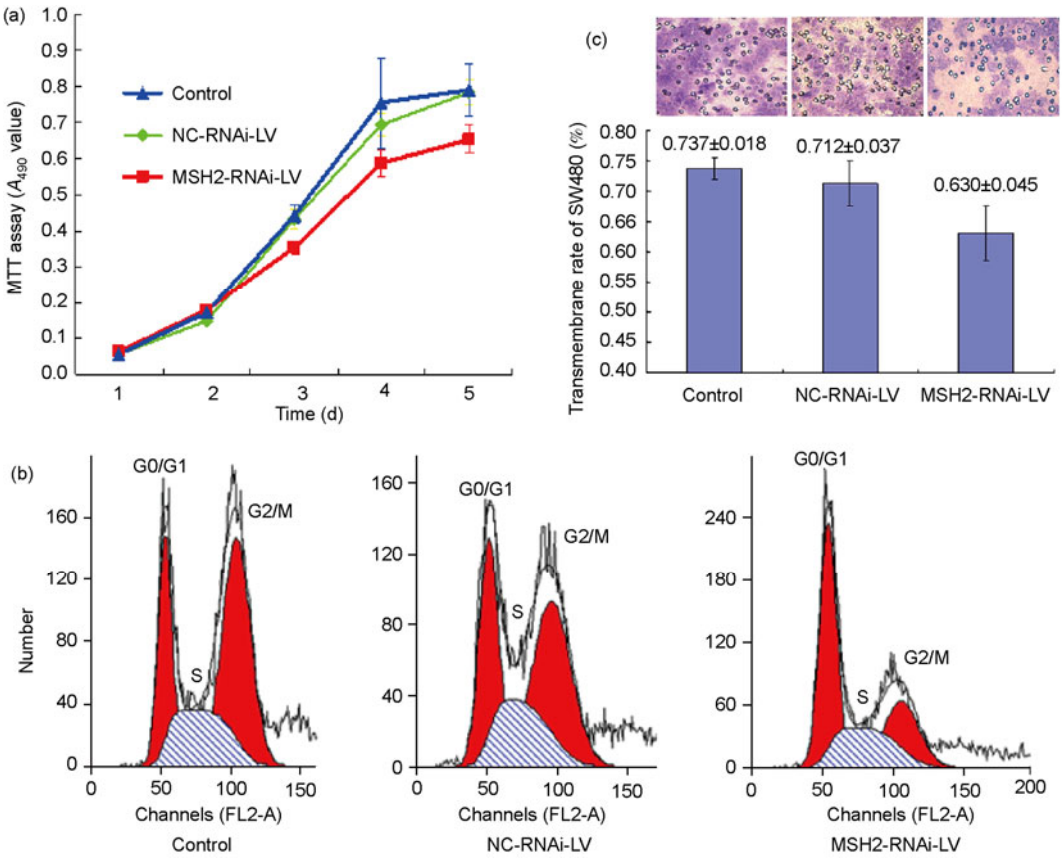

Figure 2 RNAi-mediated inhibition of *MSH2* expression in cultured SW480 cells induced decreased proliferation, G0/G1 phase arrest and decreased invasiveness. (a) Quantification of SW480 cell survival, as determined by MTT assays ($n = 3$; $P < 0.05$). Error bars represent the SEM. SW480 cell viability was reduced 48 h after infection with the MSH2-RNAi lentiviral vector. (b) G0/G1 cell cycle arrest 5 d after infection with the MSH2-RNAi lentiviral vector relative to uninfected control cells, as determined by flow cytometry (top). Data are shown as mean ± SEM. (c) Cell invasiveness was determined by transwell assays. Cells stained with crystal violet (purple) represent invasive cells. The transmembrane invasion rate of SW480 cells was calculated as the ratio of purple cells/total cells (measured spectrophotometrically at 570 nm).

Table 3 Cell survival in colon cancer cells after RNAi-mediated silencing of *MSH2* expression (flow cytometry) [a)]

	Control	NC-RNAi-LV	MSH2-RNAi-LV	*P*
G0/G1 (%)	20.92±4.89	29.33±2.28	48.49±2.18	0.000
G2/M (%)	44.20±8.51	47.18±3.87	24.81±1.10	0.005
S (%)	48.49±2.18	23.48±1.63	26.69±1.45	0.113

a) $P < 0.05$.

Transwell assays were carried out to determine the invasiveness of SW480 cells following RNAi-mediated inhibition of *MSH2*. Five days after RNAi-mediated *MSH2* silencing, SW480 cells exhibited decreased rates of cell invasiveness, as determined by the amount of transmembrane crystal violet (CV) staining, which was measured spectrophotometrically (Figure 2(c), Table 4). The results of this experiment showed that decreased *MSH2* expression was associated with decreased cell invasiveness, and suggested that *MSH2* may positively regulate cell invasion.

3 Discussion

In the current study, a simulated HNPCC cell model was established by knocking down *MSH2* expression in the colon cancer cell line, SW480, using shRNA-mediated interference (RNAi). Van Oosten et al. [12] used *MSH2*$^{-/-}$ mice and derived keratinocytes to show that MSH2 plays a role in the generation of UVB-induced arrested cells: MSH2 deficiency lowered the percentage of arrested cells both *in vivo* (40%–50%) and *in vitro* (30%–40%). UVB-induced apoptosis is reduced in MSH2-deficient cells, and correlates with decreased activation of p53, suggesting that MSH2 may act upstream of p53 to induce UVB-mediated apoptosis. Chang et al. [13] examined chemosensitivity and the cell cycle response to oxidative stress in several MMR-deficient (HCT116, SW48 and DLD1) and MMR-proficient (CaCo2, SW480 and HT29) colon cancer cell lines. RNAi-mediated gene silencing has been used in recent studies, for example Wei et al. [14] reported the silencing of STAT4 by siRNA in mouse lymphoma cell line (EL-4).

MSH2 has been shown to play a role in cell proliferation. In our study, RNAi-mediated *MSH2* silencing in SW480 cells led to decreased cell proliferation. Srivastava et al. [6] reported significantly higher levels of MSH2 protein in 28 glioblastoma multiforme samples relative to 27 unpaired astrocytoma samples; however no such difference in MLH1 levels was observed. Bae-Jump et al. [15] reported rapamycin increased cisplatin-induced apoptosis and stimulated expression of MSH2 and MSH6 in endometrial cancer cell lines, which showed that MSH2 correlated with cell proliferation. In tissues with high proliferative activity, such as digestive mucosa and endometrium, an increase in MSH2 protein levels may be required to protect cells from DNA replication errors [16].

Table 4 Cell invasiveness of colon cancer cells after RNAi-mediated silencing of *MSH2* expression (Transwell assays)[a)]

	Control (*A*)	NC-RNAi-LV (*A*)	MSH2-RNAi-LV (*A*)	*P*
Transmembrane rate	0.737±0.018	0.712±0.037	0.630±0.045	0.023

a) $P < 0.05$.

MSH2 binds to CHK1 and CHK2 both *in vivo* and *in vitro* [17]. ATM (ataxia-telangiectasia-mutated kinase), CHK1 and CHK2 have all been implicated in triggering the G2/M checkpoint response, and MMR genes are presumed to play a role in the G2/M cell cycle checkpoint [18]. Campregher et al. [19] used an *in vitro* co-culture model that mimicked intestinal inflammation in ulcerative colitis, in which the activated neutrophils caused an accumulation of target cells in G2/M, which was consistent with the installation of a DNA-damage checkpoint. Cells that did not express MSH2, p53 or p21$^{waf1/cip1}$ failed to undergo the G2/M arrest. They provided molecular evidence for an MSH2-dependent G2/M checkpoint arrest and for the presence of replication errors. Following UV irradiation, p53 activates the transcription of the MMR gene, *MSH2*. Evidence suggests that MSH2 and p53 may provide positive feedback regulation for the DNA damage response after UV irradiation, and that MSH2 may also function in UV-induced G1 arrest [20]. In our study, RNAi-mediated MSH2 silencing in SW480 cells led to a cell cycle arrest at the G0/G1 phase, providing evidence for a role of MSH2 in the G1/S checkpoint. MSH2 plays an important role in cell cycle control.

HNPCC patients, who often carry inactivating mutations in the *MSH2* gene, have better survival rates than sporadic CRC patients [21]. Nijhuis et al. [22] found that the absence of MSH2 expression is associated with a high-risk profile in early stage cervical cancer. However, in our study, colon cancer cell invasiveness was decreased in cells subjected to RNAi-mediated inhibition of MSH2 expression. These findings provide insights into the molecular basis of the clinical features of HNPCC. Collectively, our results suggest that MSH2 may serve as a potential therapeutic target for gene therapy-based approaches to colon cancer, thus establishing a basis for future clinical studies aimed at the treatment of CRC patients.

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